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Chromosomal Localization and Restriction Fragment Length Polymorphism Analysis of Annexins III, IV, and V

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Abstract

Chromosomal Localization and Restriction Fragment Length Polymorphism Analysis of Annexins III, IV, V

by Donald Alan Frankenberry

Chairperson of the Supervisory Committee:
Assistant Professor Jonathan F. Tait
Department of Laboratory Medicine

The annexins are a family of recently identified calcium-dependent phospholipid binding proteins with preference for anionic phospholipid. Chromosomal mapping of the genes for annexins III, IV, and V was undertaken as part of a study of their structure and function. The genes for annexin III and annexin V were localized to human chromosome 4 by utilizing a panel of human-hamster somatic cell hybrids and the polymerase chain reaction to amplify intron containing regions of these genes. Verification of these two localizations was performed using a human-mouse hybrid cell line containing human chromosome 4 as its only human DNA complement. Verification of the localization of annexin III was also performed using Southern blot analysis of genomic DNA specimens from the hybrid cell panel digested with Hind III. The annexin IV gene was not definitively localized in this study. Restriction mapping of these three genes suggests that they are of moderate size, 20 kb to 50 kb. The following restriction enzymes were used to screen for restriction fragment length polymorphisms at these loci: Bam HI, Bgl II, Eco RI, Hind III, Msp I, Pst I, Pvu II,

Rsa I, Taq I. Restriction fragment length polymorphisms were identified at the annexin V locus with Taq I and Pvu II, and at the annexin III locus with Hind III and Bgl II. Polymorphisms were not detected for annexin IV with Bam HI, Bgl II, Eco RI, Hind III, Msp I, Pst I, Pvu II, Rsa I, and Taq I.

Chromosomal Localization and Restriction Fragment Length Polymorphism Analysis of Annexins III, IV, and V

by

Donald Alan Frankenberry

A thesis submitted in partial fulfillment
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

complementary DNA (DNA equivalent of final mRNA).....cDNA

deoxyribonucleic acid.....DNA

deoxynucleotide triphosphates.....dNTPs

ethylenediaminetetraacetic acid.....EDTA

placental anticoagulant protein.....PAP

polymerase chain reaction.....PCR

random hexanucleotide primers.....p(dN)6

restriction fragment length polymorphism.....RFLP

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Special thanks to Kathy Clayson for her support and enthusiasm during the two years of this academic endeavor. I would like to express particular appreciation to Dr. Jonathan Tait for his unwavering guidance, infinite patience, and impeccable expertise. A heartfelt thanks to Don Gibson for preparation of oligonucleotides, Sheyla West for DNA specimens, and the rest of the Genetics Laboratory team for their assistance during my project. I would also like to recognize Dr. Marie Coyle and Ms. Joyce Behrens for serving on my thesis committee. Last but not least, my wife Cindy deserves special thanks for her continual support throughout this project.

CHAPTER I

Introduction

Annexin Proteins

The annexins (Crumpton & Dedman, 1990) are a family of recently identified calcium-dependent phospholipid binding proteins with preference for anionic phospholipid (reviewed by Klee, 1988; Crompton et al., 1988a; Burgoyne & Geisow, 1989). At the present time the annexin family contains a minimum of eight members (Haigler et al., 1989; Hauptman et al., 1989). Annexins I, II, III, IV, V, VII, and VIII contain four copies of an 80-amino acid repeating unit originally identified in lipocortin/annexin I (Wallner et al., 1986). Annexin VI contains eight copies of the 80-amino acid repeating unit (Sudhof et al., 1988; Crompton et al., 1988b).

Several groups have identified members of the annexin family based on their in-vitro anticoagulant activity (Reutelingsperger et al., 1985, 1988; Maurer-Fogy et al., 1988; Hauptmann et al., 1989; Iwasaki et al., 1987; Funakoshi et al., 1987a, 1987b; Tait et al., 1988, 1989). At the University of Washington, four members of the annexin

family were isolated and named PAP I, II, III, and IV (Funakoshi et al., 1987a; Tait et al., 1988), corresponding to annexins V, IV, III, and II. Annexins are potent in-vitro anticoagulants and could be important in physiologic or pharmacological control of blood coagulation. However, the involvement of annexins in control of blood coagulation in-vivo is so far unproven (Flaherty et al., 1990). In addition to their anticoagulant properties, the annexins have been identified by investigators as possible mediators of exocytosis, regulators of inflammation, substrates for protein tyrosine kinases, mediators of membrane-cytoskeletal linkage, inhibitors of phospholipase A2, and mediators of intracellular calcium signaling (Haigler et al., 1989; Burns et al., 1989; Crompton et al., 1988b; Kaetzel et al., 1989; Burgoyne & Geisow, 1989; Klee, 1988). Most recently, annexin III has been identified as inositol 1,2-cyclic phosphate 2-phosphohydrolase, an enzyme of inositol phosphate metabolism (Ross et al., 1990).

Previous Annexin Chromosomal Localization

At the start of this research project, two members of this protein family had been localized. Annexin I was localized to a single locus in the region 9q11-q22 (Huebner et al., 1988). Annexin II was localized to four loci: 4q21-q31.1; 9pter-q34; 10q proximal to 10q24; and

15q21-15q22 (Huebner et al., 1988). During the course of this project annexin VI has been localized to 5q32-q34 (Davies et al., 1989), and annexin V to 4q28-q32 (Modi et al., 1989). The remaining members of the annexin family have not yet been mapped to their chromosome of origin.

Chromosomal Localization

Determining the location of genes encoding proteins is essential for the diagnosis of genetic disease and the development of potential cures via gene therapy (Watson et al., 1983). Localization of the annexins will add to the stock of available genetic markers for general use in genetic mapping. Chromosomal localization of the genes for the production of the annexins is also an integral part of the study of their physiologic role and structure.

Somatic cell hybrids are commonly used in chromosomal localization analysis (Ephrussi, 1972; Ringertz & Savage, 1976). Somatic cell hybrids are produced from the fusion of human cells and cells from another species, commonly a rodent species. Each somatic cell hybrid contains a full complement of rodent chromosomes with the addition of one or more human chromosomes. The complement of human chromosomes in each hybrid cell line is normally identified by karyotype (Harris & Watkins, 1965; Ephrussi & Weiss, 1965).

Chromosomal localization using somatic cell hybrids can be performed utilizing DNA probes specific for the gene of interest. This is usually done by Southern blot analysis.

Labelled DNA probe hybridizes to genomic DNA samples from the hybrid cells digested with selected restriction enzymes. The restriction enzymes are selected on the criteria that they produce unique size human fragments significantly different than the size of the fragments produced with the rodent genomic DNA. The identification of the characteristic human restriction fragment pattern in a panel of hybrid cell lines, each containing one or more human chromosomes, enables the investigator to determine the chromosome(s) most likely to contain the gene of origin.

Another major method of chromosomal localization is in-situ hybridization. This technique utilizes the hybridization of labelled probe to metaphase chromosomal spreads of human cells (Cannizzaro & Emanuel, 1984; Chandler & Yunis, 1978; Gerhard et al., 1981). By examining the hybridization pattern, investigators are able to determine the chromosome(s) containing the gene(s) of interest and often their location within the chromosome.

Very recently, the polymerase chain reaction (PCR) has also been utilized in the performance of chromosomal localization using hybrid cell lines (Iggo et al., 1989, Abbott et al., 1989; Dionne et al., 1990). PCR is a technique which allows the exponential amplification of a specific nucleic acid sequence. PCR amplification utilizes two oligonucleotide primers that span the DNA sequence to be amplified. With repeated cycles of DNA denaturation, annealing of primers to their complementary sequences, and

extension of the annealed primers with DNA polymerase, an exponential increase in the target sequence is realized (Saiki et al., 1985; Mullis & Faloona, 1987; Ehrlich, 1989). Chromosomal localization studies using PCR and cell hybrids utilize primers designed to be complementary to coding or non-coding regions in or around the DNA sequence of interest. Hybrid cell lines producing the characteristic human PCR product contain the chromosome(s) containing the gene(s) for the protein of interest. Chromosomal localization by this technique can be performed by the amplification of coding (exon) sequence (Dionne et al., 1989). Somatic cell lines positive for the PCR product can be inferred to contain the chromosome(s) of origin.

An alternative to chromosomal localization via amplification of exon sequence is localization based on the amplification of intron sequence (Iggo et al., 1989; Abbott et al., 1989). PCR primers are designed complementary to regions of the cDNA sequence that span intron sequence. Since intron sequence is not translated into the final protein, it is generally less highly conserved than exon sequence through evolution. Primers amplifying PCR products larger than the corresponding cDNA product are assumed to span intron sequence (Figure 1). This difference in the size of the amplified products between species is the basis of chromosomal localization via amplification of intron sequence (Iggo et al., 1989).

Restriction Fragment Length Polymorphisms (RFLPs)

RFLPs are a result of variation in the DNA sequence among individuals. RFLPs are detected via Southern blot analysis of human genomic DNA cleaved with restriction enzymes and hybridized to DNA probes specific for the gene product of interest (Kan & Dozy 1978; Donis-Keller et al., 1986; Weatherall, 1985).

The identification of RFLPs serve an important function in the process of mapping the entire human genome. It is possible to arrange these RFLPs, representing specific loci, by use of mathematical analysis and their physical location into a human genomic map (Drayna, 1986; Donis-Keller et al., 1987; Nakamura et al., 1987).

RFLPs closely associated with or linked to an abnormal inheritable phenotype (DNA marker loci) are very useful provided they can be followed through families afflicted by the disease. The use of linkage analysis allows the mapping of the gene(s) responsible for the disease with respect to the DNA marker loci, without necessity for exact characterization of the gene responsible (Botstein et al., 1980). For example, the genes for Huntington's disease and cystic fibrosis have been localized by this type of linkage analysis (Gusella et al., 1983; Tsui et al., 1985; White et al., 1985; Wainwright et al., 1985).

Thesis Objectives

The first objective of this research project was the chromosomal localization of the genes for annexins III, IV, and V (PAP III, II, I). The second objective was the detection of possible RFLPs at the gene loci for these three annexins. RFLPs discovered will be useful for general genomic mapping and identification of genes causing disease. In addition, discovery of a disease linked to an annexin gene would be an important finding possibly clarifying the physiologic role of the annexins.

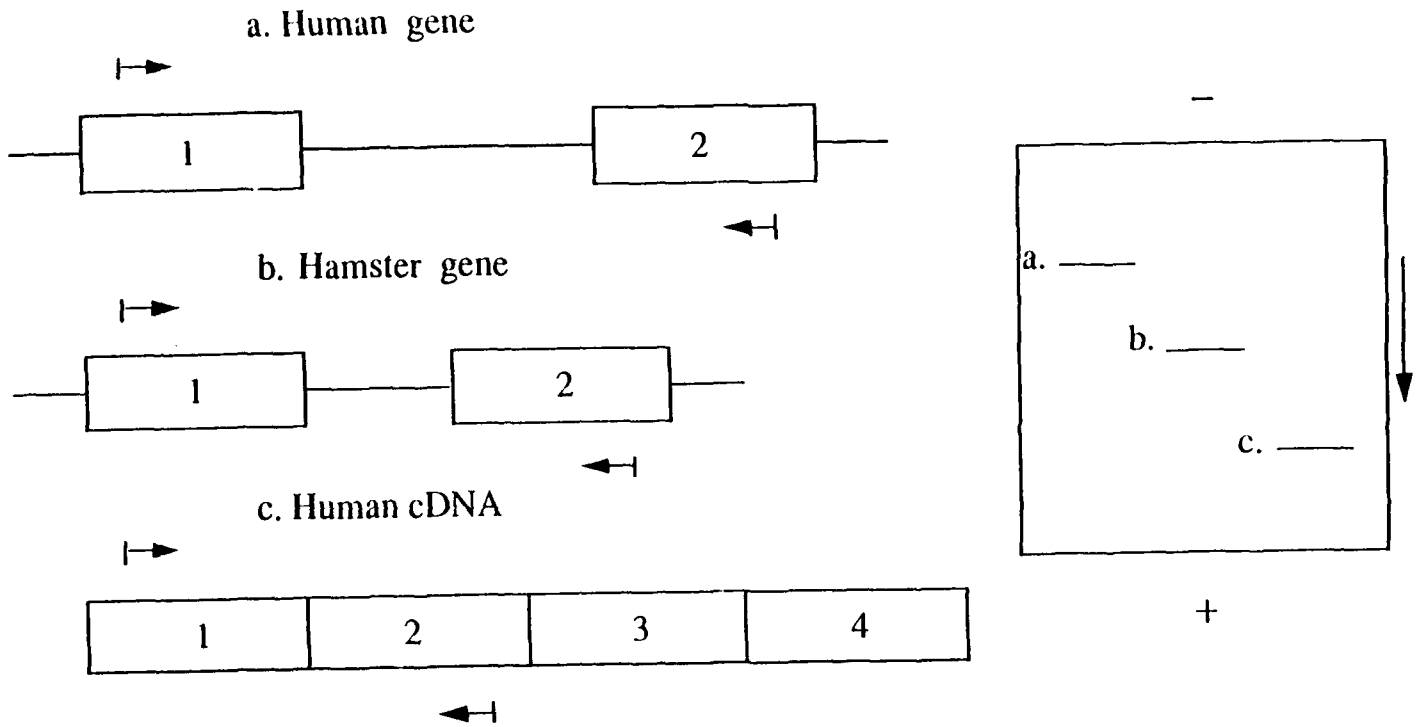

Figure 1. Chromosomal Localization Based on PCR Amplification of Intron Sequence. PCR primers designed according to cDNA sequence produce amplified product matching distance between primer annealing sites. PCR amplification of a hamster gene containing intron sequence between primer annealing site produces larger PCR product. Human gene containing larger intron sequence between primer annealing sites produces the largest product.

CHAPTER II

Methods and Materials

Materials

Taq DNA polymerase (AmpliTaq, Perkin-Elmer Cetus, Emeryville, CA).

Proteinase K (Sigma Chemical Company, St. Louis, MO).

Ultrapure dNTP set (Pharmacia Technology Inc., Piscataway, NJ).

Mouse genomic DNA, prepared from male strain C57BL/6 (Gift from Dr. David Adler).

Human-hamster somatic cell hybrid DNA (Bios Corporation, New Haven, CT).

SeaKem GTG, ultra pure agarose (FMC BioProducts, Rockland, ME).

[alpha-^{32}p]-dCTP, specific activity 3,000 Ci/mmol (product # NEG-013H, New England Nuclear Research Products, DuPont, Wilmington DE.)

Random hexanucleotide primers, p(dN)6 (Pharmacia Biotechnology Inc., Piscataway, NJ).

Klenow fragment of DNA Polymerase I (product # 104531, Boehringer Mannheim, Indianapolis, IN).

Restriction endonucleases (Boehringer Mannheim, Indianapolis, IN).

Sephadex G-50 column (Nick Column DNA grade, Pharmacia Technology Inc., Piscataway, NJ).

Gene Screen Plus nylon transfer membranes (DuPont Biotechnology Systems, Boston, MA).

X-ray film XAR-2 (Eastman Kodak, Rochester, NY).

DuPont Cronex Lightning Plus GL intensifying screens (DuPont Biotechnology Systems, Boston, MA).

Mineral oil (Sigma Chemical Company, St. Louis, MO).

Methods

PCR Primers

Oligonucleotides were synthesized using an Applied Biosystems Model 381A DNA Synthesizer utilizing phosphoramidite chemistry and reagents obtained from the manufacturer. The stepwise yield was usually > 99.0% and the oligonucleotides were used without further purification.

PCR Reactions

PCR reactions were performed as described by Mullis & Faloona (1987) and Saiki et al. (1988). The reactions were prepared in sterile 0.6 mL microcentrifuge tubes with dedicated pipettes. Each 50 uL reaction contained 100-500 ng of genomic DNA, 1.0 U Taq polymerase, 200 uM of each dNTP, 10 pmol of each primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1 g/L gelatin. Reactions contained 1.5 mM $MgCl_2$ except where noted otherwise. Each reaction was overlayed with 40 uL of mineral oil. Amplification was performed on the DNA Thermal Cycler (Perkin-Elmer) using step cycle profile methodology. Each PCR reaction included a 94^{o}C x 6 min denaturation step preceding amplification and a 72^{o}C x 10 min extension period following the amplification.

Electrophoresis of PCR Products

Amplified PCR product (15 uL) was electrophoresed for 70 V x 4 h on a (11 cm X 14 cm X 8 mm) 1.4% agarose gel in 0.089 M Tris base, 0.089 M boric acid, and 0.002 M EDTA, pH 8.0. Molecular weight markers consisted of a Hind III digest of phage lambda DNA and phi-X174 DNA digested with Hae III. Agarose gels were stained with 1.2 ug/mL ethidium bromide in H_20 for 10 min, destained in H_20 for 20 min, and photographed under 302 nm transillumination.

DNA Preparation

Human genomic DNA was isolated from nuclei of white cells following the method described by Kunkel et al. (1977) and modified by Bell et al. (1981). Red blood cells were lysed by mixing 3 to 5 mL of EDTA anticoagulated whole blood with an equal volume of 2X lysis buffer containing 0.6 M sucrose, 20 mM Tris base pH 7.0, 10 mM $MgCl_2$, and 3% Triton X-100. An equal volume of 1X lysis buffer was added; the tube was then mixed and placed on ice for 10 minutes. The mixture was inverted several times during the incubation on ice, centrifuged, and the supernatant was removed leaving the white blood cell nuclei in pellet form.

Proteinase K digestion was performed by the addition of 3.5 mL of freshly prepared proteinase K lysis buffer containing 0.01 M NaCl, 0.01 M Tris-HCl pH 7.6, 0.01 M EDTA, 0.5% sodium dodecyl sulfate, and proteinase K at a minimum concentration of 200 ug/mL. The reaction mixture was

incubated at 37°C overnight or until all visible clumps of white blood cells were eliminated.

DNA extraction was performed with the addition of 2.0 mL of phenol saturated with 10 mM Tris-EDTA, pH 7.6, and 2.0 mL of chloroform. This procedure was repeated twice on the aqueous layer. The last extraction was performed with 2 mL of chloroform.

Precipitation of the DNA from the aqueous layer proceeded with the addition of 4.0 M NaCl to a concentration of 0.4 M NaCl. The DNA was precipitated with the addition of 2 to 2.5 volumes of 100% ethanol. The mixture was placed at -20°C overnight, centrifuged, and the ethanol removed. The saved precipitate was dried under vacuum and resuspended in 200 uL of buffer containing 10 mM Tris-HCl pH 7.6, 10 mM NaCl, and 1 mM Na_2-EDTA. The DNA concentration was determined spectrophotometrically at 260 nm using a 1 cm light path and an extinction coefficient of 0.02 mL/ug.

Purified genomic DNA samples from a panel of 25 human-hamster hybrid cell lines were obtained from the Bios Corporation. The cell lines were prepared by fusing normal human B lymphocytes from unrelated donors with Chinese hamster ovary-K1 cells. The cell lines were selected according to Carlock et al. (1986). The chromosome content of each cell line was determined by the supplier based on cytogenetic evaluation of 20 metaphase spreads (Table 1).

The HA(4A) cell line, a mouse-human hybrid containing only human chromosome 4, was obtained from Dr. Anne Killary (Baylor University).

cDNA Probes

The cDNA probe for annexin V was the plasmid pPAPI-1.6, which contains a 1459-bp insert in the EcoRI site of pUC18 (Funakoshi et al., 1987b). This cDNA contains the complete 960-bp protein coding region as well as 12 bp of 5′ non-coding sequence and 487 bp of 3′ non-coding sequence. The cDNA sequence was confirmed by restriction mapping with Eco RI, Hind III, and Pst I.

The cDNA probe for annexin IV was the plasmid pPAPII-B6, which contains a 1915-bp insert in the EcoRI site of pUC18 (Miao, C. H., unpublished). This cDNA contains the complete 963-bp protein coding region plus 12 bp of 5′ non-coding sequence and 940 bp of 3′ non-coding sequence. The sequence is identical to bases 74 to 1036 of the human PP4-X cDNA (Grundman et al., 1988) with the exception of a G in place of the A seen at position 366 in PP4-X (Grundman et al., 1988). The cDNA sequence was confirmed by restriction mapping with Dde I, Eco RI, Hind III, Pst I, and Pvu II.

The cDNA probe for annexin III was the plasmid pPAPIII-28A, which contains a 1268-bp insert in the Eco RI site of pUC18 (Miao, C.H., unpublished). This cDNA contains the complete 969-bp protein coding region and 36 bp of 5′

non-coding sequence and 263 bp of 3′ non-coding sequence. The sequence of this clone is identical to bases 11 to 1278 of the human lipocortin III cDNA (Pepinsky et al, 1988) with the exception of a T in place of the C seen at position 1057 in lipocortin III (Pepinsky et al., 1988). The cDNA sequence was confirmed by restriction mapping with Eco RI, Hinc II, Hinf I, Pst I, Pvu II, and Taq I.

Preparation of cDNA Inserts

The cDNA insert was removed from the polylinker region of pUC18 at the Eco RI cloning sites. 50 ug of plasmid was digested for two hours at 37°C with 4 U of Eco RI per ug of DNA in a total volume of 500 uL. The restriction buffer contained 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol. The restriction digest was loaded with 0.2 volume Type II loading dye containing 25% Ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol. The sample was run into an (11 cm X 14 cm X 8 mm) agarose gel (SeaKem GTG) at 100 volts for 25 minutes. Electrophoresis was performed using optimal conditions for identification of the band of interest: annexin III, 1% gel, 22 V for 16 h; annexin IV, 0.9% gel, 25 V for 18 h; annexin V, 0.9% gel, 25 V for 18 h, in 0.089 M Tris base, 0.089 M boric acid, and 0.002 M Na_2-EDTA, pH 8.0. The gels were stained with ethidium bromide as described above. The band of interest was excised from the gels. The DNA was electro-eluted from the agarose fragments using Model 422 Electro-Eluter (Bio-

Rad Laboratories). Current for elution of the three annexin cDNA fragments was constant at 10 mA per elution tube. Annexin III cDNA was eluted for 45 min, annexin IV for 58 min, and annexin V for 50 min. The DNA elution buffer contained 40 mM Tris base, 20 mM glacial acetic acid, and 1 mM 0.5 M Na_2-EDTA, pH 8.0.

The electroeluted DNA was extracted by the addition of 1 volume phenol saturated with 10 mM Tris-EDTA, pH 7.6, followed by extraction with 0.5 volume phenol plus 0.5 volume chloroform. The last extraction was performed with 1 volume of chloroform. Each extract was micro-centrifuged for 1 minute and the top layer removed. The DNA was purified by ethanol precipitation by the addition of 0.1 volume 3 M sodium acetate pH 5.2, and mixed by inversion; 2 volumes of 100% ethanol were then added, mixed, and centrifuged for 10 minutes. The precipitate was washed with 1000 uL of 70% ethanol. After a micro-centrifuge spin for 10 minutes, the saved precipitate was dried under vacuum and resuspended in 100 uL 10 mM Tris-HCl pH 8.0 and 1 mM Na_2-EDTA. The DNA concentration was determined spectrophotometrically at 260 nm using an extinction coefficient of 0.02 mL/ug and a 1 cm light path.

cDNA Probe Labelling

The cDNA fragments were then radioactively labelled with [alpha^{32}p]-dCTP using random hexanucleotide primers and Klenow fragment according to Feinberg and Vogelstein (1983).

The reaction mixture contained 50 ng of DNA probe, 2.5 uL of primers (A260 = 50), and H_20 to bring the final reaction volume to 25 uL after addition of all reagents. The reaction was heated in a boiling H_20 bath for 3 minutes, quickly centrifuged, and briefly placed on ice. The remaining reagents were then added: 2.5 uL of a solution containing dATP, dTTP, and dGTP each at a concentration of 0.5 mM; 2.5 uL of buffer containing 500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM dithiothreitol, and 500 ug/mL of nuclease-free BSA; 5 ul (50 uCi) or 10 ul (100 uCi) of [alpha^{32}p]-dCTP (specific activity of 3,000 Ci/mmol); and 1 uL of 5 U/uL Klenow fragment. This mixture was briefly centrifuged and incubated at room temperature for 2 hours. 1 uL of 0.5 M Na_2-EDTA was added to stop the reaction. Unincorporated [alpha^{32}p]-dCTP was removed from the labelled probe by gel filtration using a Sephadex G-50 column measuring 2.5 cm x 1.2 cm. The probe was added to a column pre-equilibrated with 3,000 uL of buffer and eluted with 800 uL of buffer. The radioactivity of a 5 uL aliquot of the collected eluate was then quantitated on a radioisotope counter and the specific activity calculated in dpm/ug. The majority of labelling reactions produced specific activities in excess of 1 x 10^9 dpm/ug.

DNA was transferred from agarose gels to nylon membranes using the alkaline-transfer method of Reed and Mann (1985). Electrophoretic gels were depurinated with gentle agitation for 15 to 30 min in 0.25 N HCl. DNA denaturation was accomplished with gentle agitation during a 90 min wash in 0.4 M NaOH. A nylon membrane was placed on top of each inverted gel followed by the addition of 10 pieces of Whatman 3 mm chromatography paper, the first four saturated in 0.4 M NaOH. Approximately 5 cm of paper towels were stacked on top with the addition of a glass plate and a 225-250 g weight. After blotting for a minimum of 3.5 hr the DNA was fixed to the nylon membrane by washing for 3 min in 0.4 M NaOH and 3 min in a solution of 0.03 M sodium citrate and 0.3 M sodium chloride.

The membranes were pre-hybridized at 65^{o}C for 30 min in hybridization solution containing 1% sodium dodecyl sulfate, 0.05 g/mL dextran sulfate, 0.5% non-fat powdered milk, 0.9 M NaCl, 0.005 M Na_2-EDTA, 0.1 M Tris-HCl, and 0.1 M Tris base adjusted to pH 8.0. Membranes were hybridized 18 hr in hybridization solution containing 3 X 10^6 dpm/mL of probe. Following hybridization the membranes were washed down to 300-1000 cpm on a mini monitor in a solution containing 1.5 mM sodium citrate, 15.0 mM sodium chloride, and 1% sodium dodecyl sulfate at 60^{o}C.

X-ray film was subsequently exposed to the radio-labelled membranes at -70°C, with or without intensifying screens, for optimal exposure.

Stripping of the nylon membranes was performed to allow investigation with mulitple cDNA probes. Membranes were washed in 200 ml of 0.4 N NaOH at 42°C for 30 minutes with gentle agitation. Membranes were removed and immediately washed at 42°C for 30 minutes with gentle agitation in a 1.5 mM sodium citrate, 15.0 mM sodium chloride, 0.1% sodium dodecyl sulfate, and 0.2 M Tris-HCL pH 7.6. Membranes were then ready for rehybridization.

Genomic DNA Restriction Enzyme Digestion for Chromosomal Localization Studies

Human genomic DNA (9 ug), hamster genomic DNA (9 ug), and human-hamster hybrid cell lines (4 to 9 ug), were digested with Hind III (9 U per ug of DNA) for two hours at 37°C. Digested DNA samples were then electrophoresed at 25 V x 18 h on (11 cm x 14 cm x 8 mm) 0.6% agarose gel using the same buffer and molecular weight markers listed in the section "electrophoresis of PCR products". Gels were then stained with ethidium bromide and DNA transferred to nylon membranes via the Southern blot procedure listed in the previous section.

Genomic DNA Restriction Enzyme Digestion for RFLP Studies

Depending upon the experimental conditions, 5 to 15 ug of human genomic DNA was digested with 4 to 10 U of enzyme

per ug of DNA for 2 h in the buffer supplied by the manufacturer. The enzymes used in the RFLP screen were selected because they were likely to reveal RFLPs based on the experience of previous investigators. All digestions were incubated at 37^{o}C with the sole exception of digestion with Taq I at 65^{o}C. Following digestion samples were electrophoresed under desired conditions, stained with ethidium bromide as outlined above, and inspected for satisfactory digestion. Southern blots were then prepared of electrophoretic gels and hybridized with labelled cDNA probe specific for each annexin.

Table 1: Human Chromosome Content of the Human-Hamster Hybrid Cell Lines[a]

BIOS CORPORATION

Chromosome Complement of Somatic Cell Hybrid Panel — Table I

Human Chromosome	Cell Line Designation 324	423	734	750	803	860	867	940	212	507	683	756	811	983	862	909	937	854	904	967	968	1006	1049	1079	1099
1							60%										+								+
2																		+							
3		+				15%				+														+	
4					+																	55%			
5			+	d	+	+	+	+	dq	+	+	d		+	+	d	+	+	d	+	+	+	+	+	d
6						+						+				+			+						
7												+										+			
8					+								+			+				+		+			
9			+												+						+				
10						15%								+										45%	
11					15%						+												+		
12										+	45%	+							+						
13				+			+					+									+	+			+
14				+			+			+	+	45%				+	+								
15				+													+					+		10%	
16																			5%	+				+	
17													+				+								
18		+		+			+						+												
19				+		45%	+				+	+										+			+
20								+		40%		+													
21						+					+	+					+		+			+			+
22					+					25%	+														+
X					+											+					+				
Y									+	+		+							+						
Marker																									60%

Abbreviations: d - # five deleted at p15.1 - p15.2; dq - multiple deletions in 5q

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[a] reproduced from literature provided by the manufacturer.

CHAPTER III

RESULTS

General Approach to Chromosomal Localization by PCR Analysis of Cell Hybrids

This localization procedure can be divided into five basic steps: PCR primers were designed based on the known cDNA structure of each human annexin. Since the annexin gene structures are unknown, primers were designed to span entire repeating units or portions of adjacent repeating units. This was done in an attempt to produce primer pairs that span human annexin gene intron-exon boundaries and produce distinctive human-sized products in the presence of homologous hamster genes. These larger amplified products would provide initial evidence for amplification of intron containing regions of the annexin genes.

PCR conditions were optimized for each primer set and multiple human DNA specimens were tested to ensure uniform production of amplified products.

PCR reactions were then performed with hamster genomic DNA to ensure that the size of any amplified product(s) were significantly different than the size of the human product(s).

To verify that amplified products were of annexin gene origin and rule out non-specific PCR amplification, high stringency probing of Southern blots of PCR product was performed with the appropriate labelled annexin cDNA.

PCR analysis of all 25 human-hamster hybrid cell lines was then performed. This amplification was followed by Southern blot verification of all cell lines producing the human sized PCR products with labelled cDNA probe.

Chromosomal Localization of Annexin V

Four primer sets were tested for annexin V (Table 2). PCR reactions were performed as described in Chapter II with a multitude of thermal profiles (Table 3). Primers JT102 and JT104 amplified multiple products visible on ethidium bromide stained gels with a major 1.5 kb band. This 1.5 kb product was larger than the cDNA product of 232 bp. The length of the cDNA products was determined according to the distance between the primer annealing sites along the cDNA. PCR reactions were then performed with the same conditions using multiple human genomic DNA samples and hamster genomic DNA. All human samples showed the same results and the hamster DNA produced multiple amplified products, all less than 1.5 kb. Longer primers were then designed to improve the specificity of primer annealing to human DNA templates. An additional 5 and 7 bp were added to the 3′ end of primers JT102 and JT104 to produce primers JT121 and JT122. PCR conditions were then optimized using these new primers,

JT121 and JT122, and 0.5 ug of template DNA. These conditions produced a single human genomic product of 1.5 kb and multiple hamster bands less than 1.5 kb.

Chromosomal localization was then attempted using the 25 human-hamster somatic cell hybrid lines. Results showed one band of 1.5 kb amplified with human genomic DNA, a single 1.5 kb product amplified strongly in somatic cell lines 803 and 1006, and weakly in somatic cell line 867 (Figure 2). Several distinct products were amplified in hamster DNA and the human-hamster somatic cell lines, indicating amplification of hamster genes.

Southern blot verification of the amplified products was performed using 2 uL of product from human genomic DNA, hamster genomic DNA, cell lines containing the 1.5 kb band (803, 1006, 867), and a sampling of the cell lines lacking the 1.5 kb band (324, 811, 937). Annexin V cDNA (0.38 ng) and annexin III cDNA (0.38 ng) were added to the gel as positive and negative controls. Annexin V cDNA hybridized to the 1.5 kb band seen on the ethidium bromide stained gels for human genomic DNA and cell lines 803, 867, and 1006. The hamster DNA and other cell lines did not show hybridization with this probe in this region. Adequate stringency of the wash was verified by the lack of cross-hybridization with the related annexin III cDNA (Figure 3).

Analysis of the human chromosome complement in each somatic cell line indicates that the gene for annexin V is located on chromosome 4. Results show 4% discordance for

chromosome 4 and at least 15% discordance for all other chromosomes (Figure 4). This 4% discordancy with chromosome 4 comes from cell line 867 which produced weak amplification of the 1.5 kb product but was negative for chromosome 4 by cytogenetic analysis. The same discordancy has been observed with this cell line for another gene localized to chromosome 4 (Tabas & Zasloff, personal communication with Dr. J. Tait). The most probable explanation is that cell line 867 contains either a translocated portion of chromosome 4 or a small percentage of cells containing chromosome 4 which were not detected by cytogenetic analysis.

Chromosomal localization of annexin V using Southern blot analysis of Hind III digests of the somatic cell hybrids did not prove as successful. No discernible human specific bands were detectable in any of the cell lines and therefore localization by this technique was impossible.

Localization of Annexin III

Two primer pairs were designed for annexin III (Table 4). PCR reactions were performed with a multitude of thermal profiles (Table 5). After optimizing PCR conditions, primers JT114 and JT115 produced a 480 bp band from human genomic DNA larger than the corresponding cDNA product of 230 bp. However, this PCR product failed to hybridize with annexin III cDNA, indicating it was not synthesized from the annexin III gene.

Primers JT130 and JT131 were then designed and synthesized. PCR reactions produced a 2.0 kb band with human genomic DNA and no visible bands with hamster genomic DNA, presumably because the primer sequences did not match the hamster gene closely enough to allow amplification. Chromosomal localization of annexin III was performed using these primers at optimized PCR conditions. The results showed the presence of the 2.0 kb product with human genomic DNA and cell lines 803 and 1006 (Figure 5). Hamster genomic DNA and the other somatic cell lines did not show any bands.

High-stringency probing of a Southern blot to verify the origin of the PCR products was performed with cell lines containing the 2.0 kb band (803, 1006), cell lines lacking the band (324, 811, 937), and the cDNA positive and negative controls. Annexin III cDNA hybridized to the 2.0 kb band seen on the ethidium bromide stained gels for human genomic DNA, cell line 803, and 1006. Hamster genomic DNA and the other cell lines did not show hybridization with this probe in this region. Adequate stringency of the final wash was verified by the lack of cross-hybridization with the related annexin V probe (Figure 6).

Chromosomal localization of annexin III using Southern blot analysis of Hind III digests of the somatic cell hybrids confirmed results obtained with the PCR methodology. Human-specific restriction fragments were seen of 9.4, 6.0, 5.6, 5.0, 4.2, 3.9, and 3.3 kb. These same human-specific bands were seen in cell lines 803 and 1006. They were

absent in hamster genomic DNA and all other cell lines (Figure 7).

Analysis of the human chromosome complement in each somatic cell line indicates that the gene for annexin III is located on chromosome 4. Results for annexin III show 0% discordance for chromosome 4 and at least 15% discordance for all other chromosomes (Figure 4).

Additional Verification of Chromosomal Localization for Annexins III and V

Verification of chromosome 4 as the location of the genes coding for annexin III and V was performed utilizing a mouse-human hybrid cell line containing chromosome 4 as its only human chromosome (Figure 8). PCR reactions were performed for both annexins as outlined earlier. An ethidium bromide stained gel of PCR products amplified with this cell line shows the characteristic 2.0 kb human-specific band produced in annexin III PCR reactions, the characteristic 1.5 kb human-specific band produced in annexin V PCR reactions, and the amplification of a 0.9 kb product in the mouse genomic specimen and the mouse-human cell line with annexin V PCR reactions. This 0.9 kb product is most likely produced from the mouse annexin V gene. These results, using a different rodent species and alternate source of hybrid DNA verify earlier results obtained with the human-hamster somatic cell hybrids.

Chromosomal localization of annexin IV via PCR amplification of intronic sequences was initiated with the design and synthesis of multiple primer pairs (Table 6). Two primer pairs produced amplified products with a denaturing temperature of 94°C and 30 cycles (Table 7). Initial PCR reactions with primers JT124 and JT140 generated products of 190 bp and 560 bp. High-stringency probing of a Southern blot containing the products with annexin IV cDNA was performed with no hybridization to either product, indicating that these two products were not of annexin gene origin. Primers JT128 and JT129 produced very faint, multiple bands less than 1.4 kb. Due to the small amount of these products produced, as visualized on ethidium bromide stained gels, these primers were not further tested. PCR reactions were then performed using all designed primer pairs with a denaturing temperature of 96°C and 40 cycles. A few small bands were produced with multiple primer pairs, while primers JT124 and JT140 produced a 3.5 kb product (Table 7). High-stringency probing of a Southern blot containing the products with annexin IV cDNA was performed with positive hybridization to the 3.5 kb band. Repeating the above PCR reaction after adjusting the denaturing temperature to 94°C produced the same 3.5 kb product.

PCR was then performed with primers JT124 and JT140 with the 25 human-hamster somatic cell hybrid cell lines using a thermal cycle of denaturing 94°C x 1 min, annealing

55°C x 1 min, and extension 72°C x 3 min. Results showed the absence of the 3.5 kb product in any of the cell lines and multiple hamster bands all significantly smaller than 3.5 kb. The absence of the band in the somatic cell hybrids could be due to the absence of the gene, mutation of the gene preventing correct primer annealing or amplification, or inhibitors present in sufficient concentrations in the DNA to inhibit the activity of Taq polymerase. A Mg^{2+} titration was performed repeating the PCR reaction with human and hamster genomic DNA with Mg^{2+} concentrations of 1.5 mM, used in all previous work, and 2.0, 2.5, 3.0 and 4.0 mM. The 3.5 kb human band and all hamster bands were greatly enhanced with 2.0 mM and more concentrated $MgCl_2$.

Chromosomal localization was again attempted using 2.0 mM $MgCl_2$. Ethidium bromide stained gels indicated the presence of a weak 3.5 kb product in cell line 854. High-stringency probing of Southern blots of these PCR products from all 25 cell lines was performed with appropriately labelled annexin IV cDNA. Cell line 854 showed two positive, closely spaced bands of 3.5 and 3.3 kb. Cell line 983 contained a positive band at 3.5 kb and cell line 904 displayed a weak band at 3.5 kb. Percent discordancy for each of the chromosomes ranged from 8% to 76%. Chromsome 2 was 8% discordant, followed by chromosome 10 at 16%. No chromosome of origin for the annexin IV gene can be definitely assigned based on this set of PCR experiments.

Chromosomal localization of annexin IV using Southern blot analysis of Hind III digests of the somatic cell hybrids proved unsuccessful in determining the chromosome of origin. Hind III was selected on the basis of producing, when probed with annexin IV cDNA, strong human-specific bands easily distinquished from the hamster pattern. Human-specific bands were seen of 10.5, 2.9, 2.7, 2.2, 2.0 and 1.7 kb. These bands were only visible in human genomic DNA. They were not present in hamster genomic DNA or any of the hybrid cell lines. Two bands, 6.2 and 4.9 kb, were shared between human DNA, hamster DNA, and the hybrid cell lines. Since no human-specific bands were seen in any of the hybrid cell lines, annexin IV was not localized by this method. However, the absence of the signal suggests that the annexin IV gene is not on a chromosome that is well represented in the hybrid cell panel.

Restriction Mapping and RFLP Analysis of the Genes for Annexin III, IV, & V

Southern blot analysis of human genomic DNA was performed in order to detect possible RFLPs and to establish the sizes and restriction maps of the annexin genes. In each case the genes are of moderate size, approximately 20 to 50 kb (Tables 8, 9, & 10).

Human genomic DNA from approximately 10 unrelated Caucasians was screened for the presence of RFLPs with 9 restriction enzymes commonly used in the genetics

laboratory. A complete listing of enzymes used and restriction fragments produced with each annexin cDNA probe is found in Table 8 (annexin III), Table 9 (annexin IV), and Table 10 (annexin V). A synopsis of the conditions used in the RFLP screen is found in Table 11. Screening with the cDNA for annexin V, RFLP systems were detected using Taq I and Pvu II (Table 12). Review of the annexin V, Eco RI restriction fragment pattern, shows a probable additional RFLP. This RFLP was recently identified and brought to our attention by another investigator (Murray, J., personal communication with Dr. J. Tait). RFLP systems were detected with annexin III using Bgl II and Hind III (Table 13). No RFLP systems were detected with annexin IV.

Inheritance patterns for three of the four RFLPs were investigated by family studies. In each case, the RFLP was inherited as a Mendelian trait. The annexin III-Hind III RFLP was not extensively investigated due to the extremely low frequency of the low molecular weight allele producing the 0.9 kb fragment. A Southern blot of a representative family, from the four families studied (12 meioses), is included for the annexin V-Taq I RFLP (Figure 9). Five families (24 meioses) were examined and a Southern blot is included of the annexin V-Pvu II RFLP (Figure 10). A Southern blot of four random individuals illustrates the three possible allele combinations, seen in the three

families evaluated (14 meiosis), of the annexin III-Bgl II RFLP (Figure 11).

Table 2: Annexin V PCR Primer Characteristics

Number	Sequence (5′ to 3′)	cDNA bases	Amino Acids
JT101	AGA CCA TGG ATG CTG GAA TTG AT	502-519 (S)[a]	PDAGID
JT102	ACT AAG CTT AGT CAT CTT CTC C	961-975 (A)[b]	CGEDD
JT103	ACT AAG CTT AAG GTA TAC TTC GAA TAG A	739-756 (A)[b]	SIRSIP
JT104	AGA CCA TGG CCT ACC TTG CAG AG	757-771 (S)[a]	AYLAE
JT110	ACA AGA TGC TCA GGC TTT AT	531-550 (S)[c]	QDAQALF
JT111	TAG TTG CTC TAA ATT GCC AG	701-720 (A)[d]	SGNLEQL
JT121	CTA AGC TTA GTC ATC TTC TCC ACA GAG C	954-975 (A)[e]	LLCGEDD
JT122	TTG AAT TCG CCT ACC TTG CAG AGA CCC T	757-776 (S)[f]	AYLAETL

[a] sense primer includes Nco I site and 2 bases at 5′ end.
[b] anti-sense primer includes Hind III site and 3 bases at 5′ end.
[c] sense primer.
[d] anti-sense primer.
[e] anti-sense primer includes Hind III site and 2 bases at 5′ end.
[f] sense primer includes Eco RI site and 2 bases at 5′ end.

Table 3: Thermal Profiles and Genomic Products Amplified with Annexin V Primers

Primers	Thermal Profile[a] D	A	E	Cycles	Genomic Product
JT101 & JT102	94°C 1 min	45°C 30 sec	72°C 3 min	30	none
JT101 & JT102	94°C 1 min	50°C 30 sec	72°C 3 min	30	none
JT101 & JT102	94°C 1 min	55°C 30 sec	72°C 3 min	30	none
JT101 & JT103	94°C 1 min	45°C 30 sec	72°C 3 min	30	none
JT101 & JT103	94°C 1 min	55°C 30 sec	72°C 3 min	30	none
JT102 & JT104	94°C 1min	45°C 30 sec	72°C 3 min	30	1.5 kb
JT102 & JT104	94°C 1 min	55°C 30 sec	72°C 3 min	30	1.5 kb
JT110 & JT111	94°C 1 min	45°C 30 sec	72°C 3 min	30	none

[a] D indicates denaturation temperature and time, A indicates annealing temperature and time, E indicates extension temperature and time.

Table 3 (continued): Thermal Profiles and Genomic Products Amplified with Annexin V Primers

Primers	Thermal Profile[a] D	A	E	Cycles	Genomic Product
JT110 & JT111	94°C 1 min	50°C 30 sec	72°C 3 min	30	none
JT110 & JT111	94°C 1 min	55°C 30 sec	72°C 3 min	30	none
JT112 & JT113	96°C 1 min	60°C 1 min	72°C 3 min	40	none
JT121 & JT122	94°C 1 min	45°C 30 sec	72°C 3 min	30	1.5 kb multiple <1.5 kb
JT121 & JT122	94°C 1 min	50°C 30 sec	72°C 3 min	30	1.5 kb
JT121 & JT122	94°C 1 min	55°C 30 sec	72°C 3 min	30[b]	1.5 kb

[a] D indicates denaturation temperature and time, A indicates annealing temperature and time, E indicates extension temperature and time.
[b] thermal profile and cycles used for chromosomal localization.

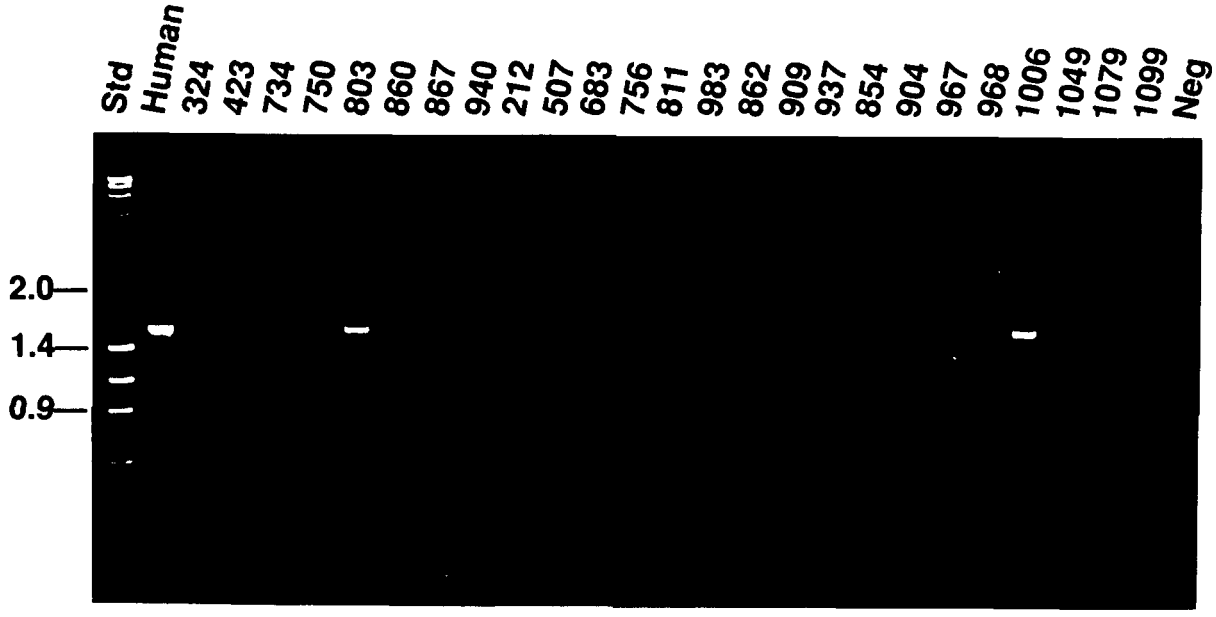

Figure 2. PCR of Analysis of Somatic Cell Hybrid Panel with Annexin V. Ethidium bromide stained gel of PCR products from 500 ng of human genomic DNA, hamster DNA, or the indicated hybrid cell line genomic DNA. PCR was performed for 30 cycles of amplification (thermal profile: 94^{o}C x 1 min, 55^{o}C x 0.5 min, 72^{o}C x 3 min) with primers JT120 and JT121. Refer to Chapter II for electrophoretic conditions. The hamster control (not shown) gave a pattern similar to cell line 734. "Neg" is the negative control (PCR reaction without added DNA).

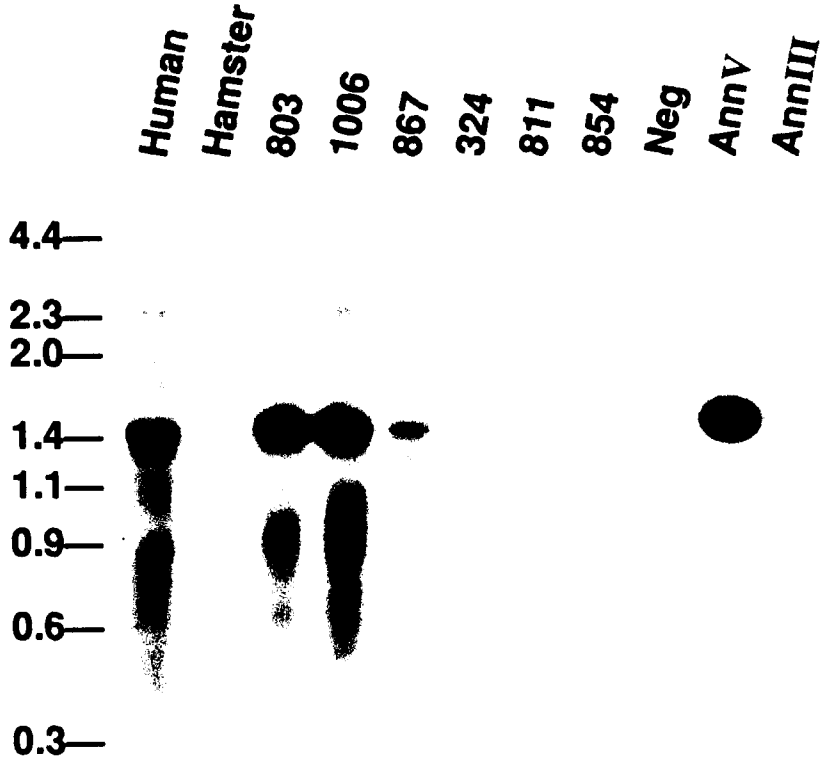

Figure 3. Southern Blot Analysis of Annexin V PCR Products. PCR product was electrophoresed at 70 V x 4.25 h on a (11 cm x 14 cm x 8 mm) 1.4% agarose gel, Southern blot performed, and hybridized with annexin V cDNA probe as described in Chapter II and III. Positive hybridization occurred with human genomic, cell lines (803, 1006, 867), and annexin V cDNA.

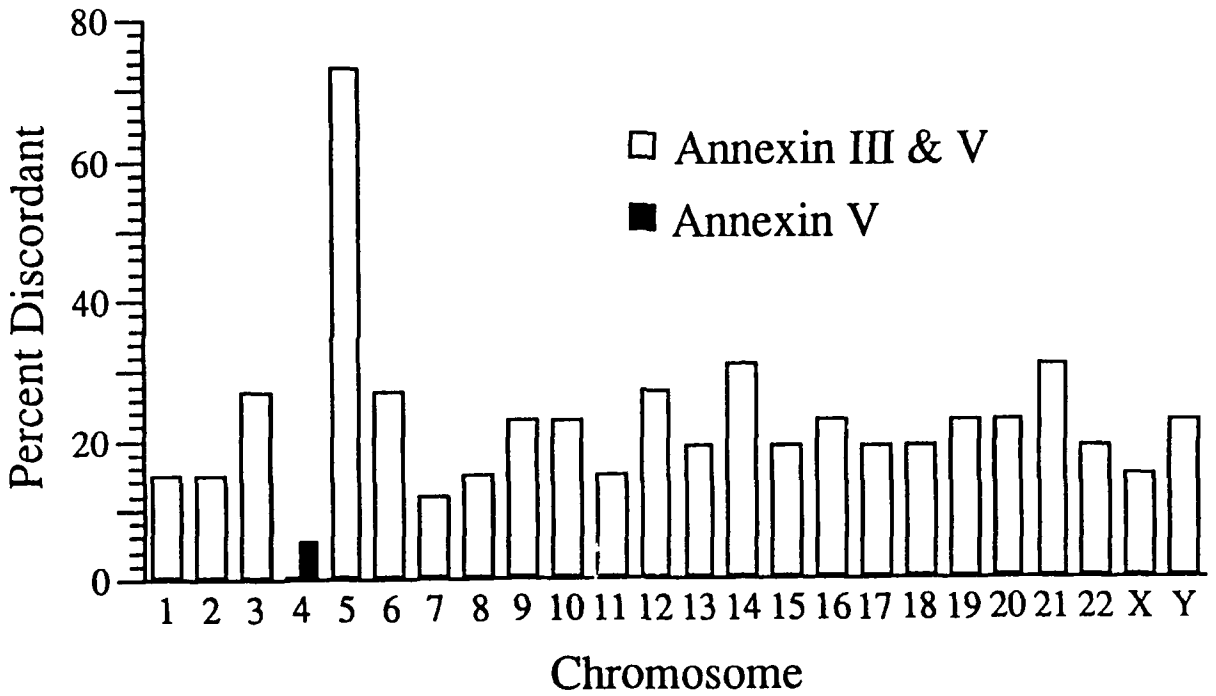

Figure 4. Discordancy Analysis for Annexins III and V. PCR localization results using the 25 human-hamster somatic cell lines for annexin III and annexin V were analyzed for chromosomal content. Percent discordancy for chromsome 4 is 0% for annexin III and 4% for annexin V. Percent discordancy for all other chromosomes was the same for annexins III and V.

Table 4: Annexin III PCR Primer Characteristics

Number	Sequence (5′ to 3′)	cDNA Bases[a]	Amino Acids
JT114	TTG AAT TCG CCT TTT TAG CAG AAA GAC T	790-809 (S)[b]	AFLAERL
JT115	GGA AGC TTT CAT CTC CAC CAC AGA TTT	986-1004 (A)[c]	KICGGDD
JT130	TTG AAT TCG AAA GTC TGA AAG TGG ATG AGC	532-553 (S)[b]	ESLKVDEH
JT131	GGA AGC TTG CTG TCC ACA ATG TCC TTT TG	697-717 (A)[c]	QKDIVDS

[a] sequence numbering based on the sequence of pPAPIII-28A.
[b] sense primer includes EcoRI site and 2 bases at 5′ end.
[c] anti-sense primer includes HindIII site and 2 bases at 5′ end.

Table 5: Thermal Profiles and Genomic Products Amplified with Annexin III Primers

Primers	Thermal Profile[a] D	A	E	Cycles	Genomic Product
JT114 & JT115	94°C 1 min	50°C 1 min	72°C 3 min	30	none
JT114 & JT115	94°C 1 min	45°C 1 min	72°C 3 min	30	none
JT114 & JT115	94°C 1 min	40°C 1 min	72°C 3 min	30	480 bp
JT114 & JT115	94°C 1 min	37°C 1 min	72°C 3 min	30	480 bp
JT114 & JT115	94°C 1 min	45°C 30 sec	72°C 2 min	30	none
JT114 & JT115	94°C 1 min	50°C 30 sec	72°C 2 min	30	none
JT114 & JT115	94°C 1 min	55°C 30 sec	72°C 2 min	30	none

[a] D indicates denaturation temperature and time, A indicates annealing temperature and time, E indicates extension temperature and time.

Table 5 (continued): Thermal Profiles and Genomic Products Amplified with Annexin III Primers

Primers	Thermal Profile[a] D	A	E	Cycles	Genomic Product
JT114 & JT115	94°C 1 min	60°C 30 sec	72°C 2 min	30	none
JT114 & JT115	94°C 1 min	37°C 1 min	72°C 3 min	30	480 bp
JT130 & JT131	94°C 1 min	45°C 30 sec	72°C 3 min	30	multiple <600 bp
JT130 & JT131	94°C 1 min	50°C 30 sec	72°C 2 min	30	2.0 kb faint <2.0 kb
JT130 & JT131	94°C 1 min	55°C 30 sec	72°C 2 min	30 35 40	2.0 kb 2.0 kb 2.0 kb
JT130 & JT131	94°C 1 min	55°C 1 min	72°C 2 min	30[b]	2.0 kb

[a] D indicates denaturation temperature and time, A indicates annealing temperature and time, E indicates extension temperature and time.
[b] thermal profile and cycles used for chromosomal localization.

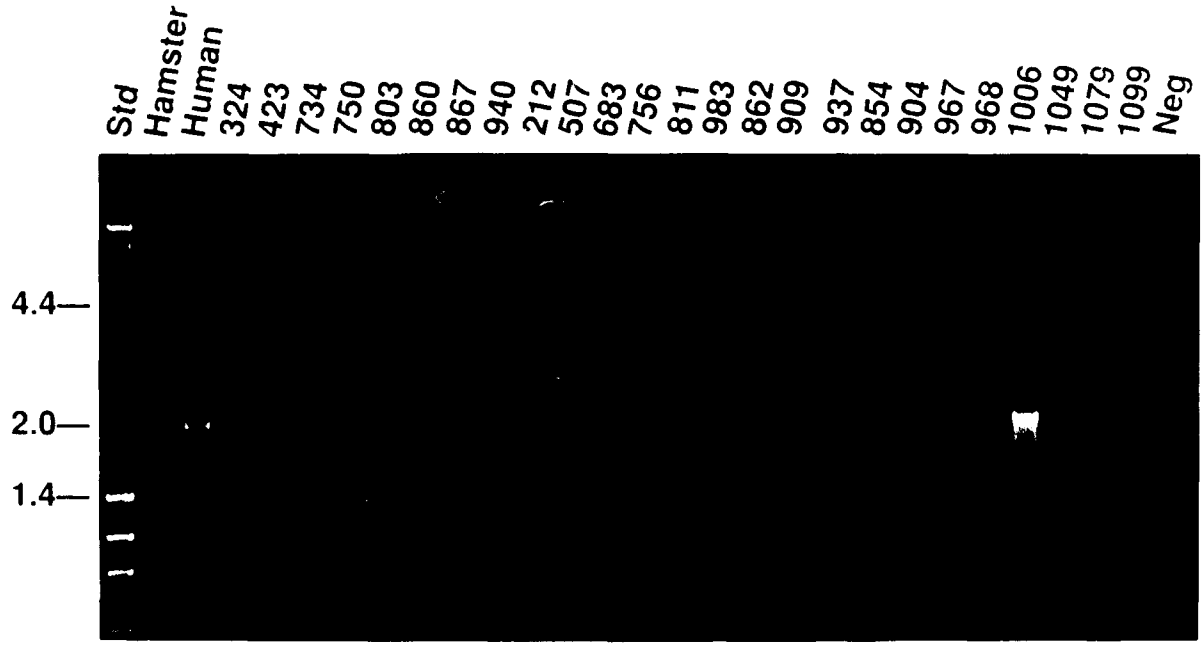

Figure 5. PCR Analysis of Somatic Cell Hybrid Panel with Annexin III. Ethidium bromide stained gel of PCR products amplified from 250 ng of human genomic DNA, hamster DNA, or the indicated hybrid cell line genomic DNA. PCR was performed for 30 cycles of amplification (thermal profile: 94°C x 1 min, 55°C x 1 min, 72°C x 2 min) with primers JT130 and JT131. Refer to Chapter II for electrophoretic conditions. "Neg" is the negative control (PCR reaction without added DNA).

Figure 6. Southern Blot Analysis of Annexin III PCR Products. PCR product was electrophoresed at 70 V x 7.5 h on a (11 cm x 14 cm x 8 mm) 1.1% agarose gel, Southern blot performed, and hybridized with annexin III cDNA probe as described in Chapter II and III. Positive hybridization occurred with human genomic, cell lines (803 & 1006), and annexin III cDNA.

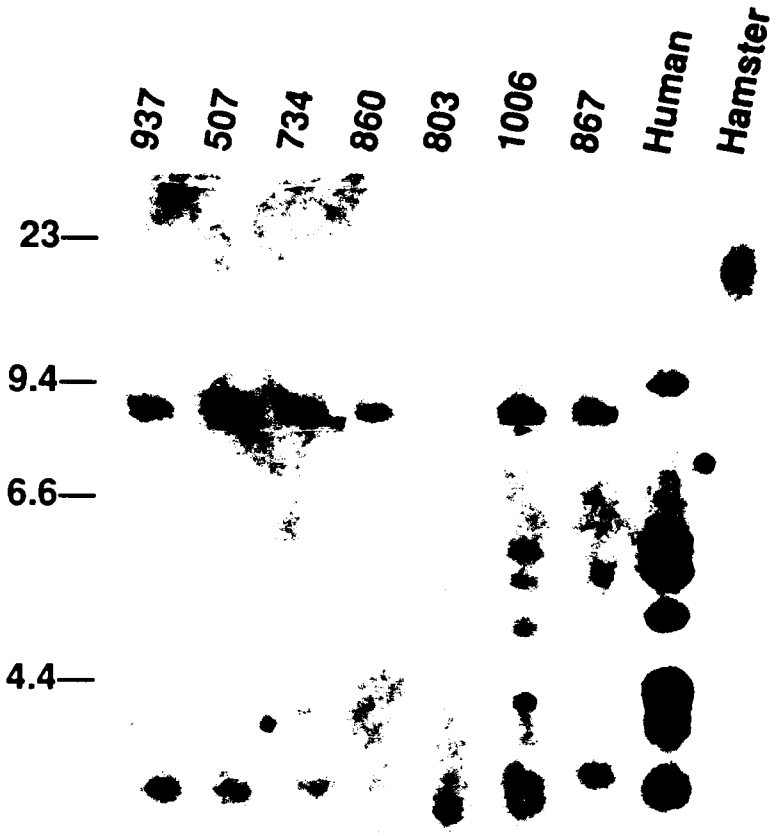

Figure 7. Chromosomal Localization of Annexin III by Southern Blot of Human-Hamster Somatic Cell Hybrids. Genomic DNA from somatic cell hybrids (4 to 9 ug), human (9 ug), or hamster (9 ug) was digested with Hind III (9 U/ug of DNA). Genomic Southern blot of human-hamster hybrids was probed with annexin III cDNA. Note the human-specific bands in cell lines 803 and 1006. Other hybrid cell lines not shown in the figure were negative for the human-specific bands.

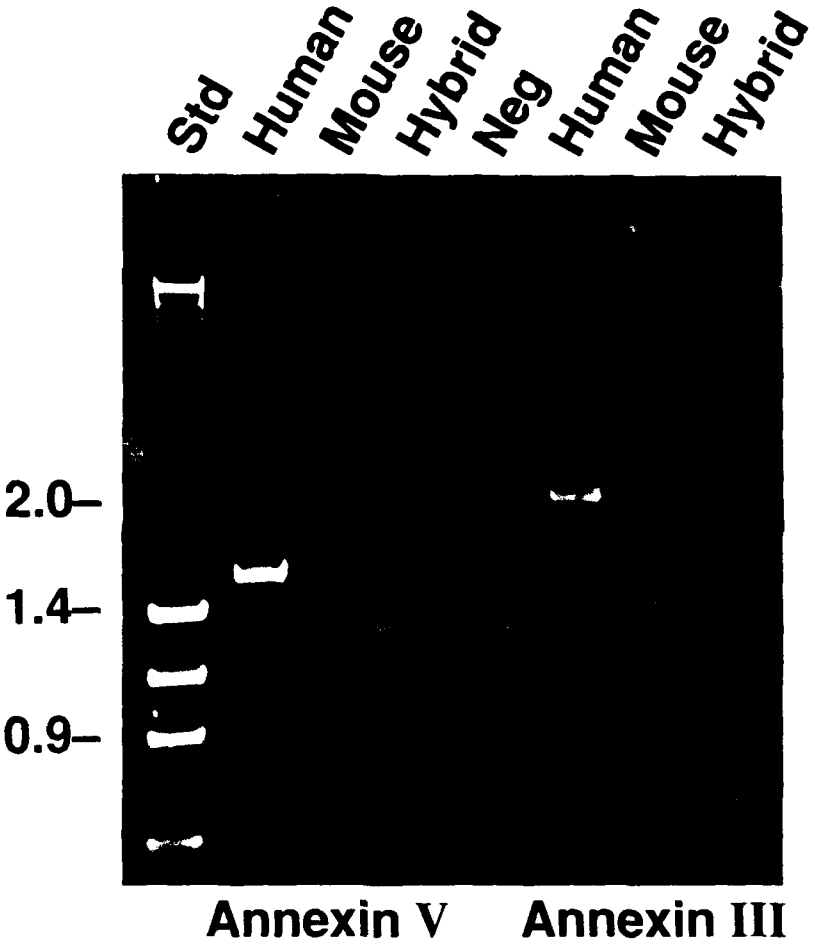

Figure 8. PCR Analysis of the HA(4A) Cell Line. This mouse-human hybrid cell line contains only human chromosome 4. Note the 2.0 kb human-specific band with annexin III, the 1.5 kb human-specific product with annexin V, and the 0.9 kb mouse-specific product with annexin V. Refer to Chapter II for electrophoretic conditions.

Table 6: Annexin IV PCR Primer Characteristics

Number	Sequence (5′ to 3′)	cDNA bases[a]	Amino Acids
JT112	ACG AAT TCA TCT GAA TAT TTT GCT GAA AAG	756-777 (S)[b]	SAYFAEK
JT113	CCA AGC TTA TTT TAA TCA TCT CCT CCA C	962-983 (A)[c]	CGGDD*N
JT124	AAG AAT TCG AAG GAA ATT ATC TGG ACG A	502-521 (S)[b]	EGNYLDD
JT125	CCA AGC TTA CTC TGT TCA ATA TCC TTC TG	667-687 (A)[c]	QKDIEQS
JT128	GGG AAT TCG ACG CCC ACG GTG CTG TAT	270-288 (S)[b]	MTPTVLY
JT129	TTA AGC TTC AAG GCT CCG TCC ATA TTG C	408-429 (A)[c]	QQYGRSLE
JT133	TTG AAT TCG CTT CAG GAT TCA ATG CCA TGG	49- 70 (S)[b]	ASGFNAME
JT134	ATA AGC TTC CTG CCG ATG GTG CTC TTG TA	184-204 (A)[c]	YKSTIGR
JT140	AAA AGC TTG GAA CAG AGA ACA GTT AGA	597-615 (A)[c]	LTVLCSR
JT141	TTA AGC TTG GAC GCT AAT AAT GGC GTC	121-139 (A)[c]	DAIISVL

[a] sequence numbering based on sequence of pPAPII-B6.
[b] sense primer includes Eco RI site and 2 bases at 5′ end.
[c] anti-sense primer includes Hind III site and 2 bases at 5′ end.

Table 7: Thermal Profiles and Genomic Products Amplified with Annexin IV Primers

Primers	Thermal Profile[a] D	A	E	Cycles	Genomic Product
JT112 & JT113	94°C 1 min	50°C 1 min	72°C 3 min	30	none
JT112 & JT113	94°C 1 min	45°C 1 min	72°C 1 min	30	none
JT112 & JT113	94°C 1 min	40°C 1 min	72°C 1 min	30	none
JT112 & JT113	94°C 1 min	37°C 1 min	72°C 1 min	30	none
JT124 & JT125	94°C 1 min	45°C 30 sec	72°C 3 min	30	none
JT124 & JT125	94°C 1 min	35°C 1 min	72°C 2 min	30	250 bp
JT124 & JT125	96°C 1 min	50°C 1 min	72°C 3 min	30	none
JT124 & JT125	94°C 1 min	45°C 30 sec	72°C 2 min	30 35 40	none none none

[a] D indicates denaturation temperature and time, A indicates annealing temperature and time, E indicates extension temperature and time.

Table 7 (continued): Thermal Profiles and Genomic Products Amplified with Annexin IV Primers

Primers	Thermal Profile[a] D	A	E	Cycles	Genomic Product
JT124 & JT140	94°C 1 min	40°C 1 min	72°C 2 min	30 35 40	560 bp 190, 560bp 190, 560bp
JT124 & JT140	94°C 1 min	35°C 1 min	72°C 2 min	30 35 40	560 bp 190, 560 bp 190, 560 bp
JT124 & JT140	94°C 1 min	45°C 1 min	72°C 1 min	30 35 40	none 560 bp 190 bp, 560 bp
JT124 & JT140	94°C 1 min	40°C 1 min	72°C 2 min	35	190 bp, 560 bp
JT124 & JT140	96°C 1 min	50°C 1 min	72°C 3 min	40	480 bp
JT124 & JT140	96°C 1 min	50°C 1 min	72°C 5 min	40	3.5 kb, 0.6 kb
JT124 & JT140	96°C 1 min	55°C 1 min	72°C 3 min	40	3.5 kb

[a] D indicates denaturation temperature and time, A indicates annealing temperature and time, E indicates extension temperature and time.

Table 7 (continued): Thermal Profiles and Genomic Products Amplified with Annexin IV Primers

Primers	Thermal Profile[a] D	A	E	Cycles	Genomic Product
JT124 & JT140	96°C 1 min	55°C 1 min	72°C 5 min	30 40	none 3.5 kb
JT124 & JT140	96°C 1 min	60°C 1 min	72°C 5 min	30 40	none none
JT124 & JT140	94°C 1 min	55°C 1 min	72°C 3 min	40[b]	3.5 kb
JT128 & JT129	94°C 1 min	45°C 30 sec	72°C 3 min	30	multiple <1.4 kb
JT128 & JT129	94°C 1 min	50°C 30 sec	72°C 2 min	30 40	faint <1.4 kb
JT128 & JT129	94°C 1 min	55°C 30 sec	72°C 2 min	30 40	none none
JT128 & JT129	94°C 1 min	55°C 30 sec	72°C 3 min	30	none
JT128 & JT129	96°C 1 min	50°C 1 min	72°C 3 min	40	520 bp, 410 bp 310 bp, 260 bp

[a] D indicates denaturation temperature and time, A indicates annealing temperature and time, E indicates extension temperature and time.
[b] thermal profile used for chromosomal localization.

Table 7 (continued): Thermal Profiles and Genomic Products Amplified with Annexin IV Primers

Primers	Thermal Profile[a] D	A	E	Cycles	Genomic Product
JT133 & JT134	94°C 1 min	45°C 30 sec	72°C 3 min	30	faint <1.4 kb
JT133 & JT134	94°C 1 min	50°C 30 sec	72°C 3 min	30	none
JT133 & JT134	94°C 1 min	55°C 30 sec	72°C 3 min	30	none
JT133 & JT134	96°C 1 min	50°C 1 min	72°C 3 min	40	480 bp, 240 bp 230 bp
JT133 & JT141	94°C 1 min	45°C 30 sec	72°C 2 min	30	none
JT133 & JT141	94°C 1 min	35°C 1 min	72°C 2 min	30	none
JT133 & JT141	94°C 1 min	40°C 1 min	72°C 2 min	30	none
JT133 & JT141	96°C 1 min	50°C 1 min	72°C 3 min	40	none

[a] D indicates denaturation temperature and time, A indicates annealing temperature and time, E indicates extension temperature and time.

Table 8: Restriction Map of Annexin III Gene

Enzyme	Bands Observed (kb)	Total kb[a]
Bam HI	20.0, 9.4, 8.9, 3.7	42.0
Eco RI	8.7, 6.6, 5.3, 4.7, 3.3, 2.5	31.1
Msp I	8.0, 7.2, 5.6, 4.5, 3.7, 2.3, 1.8	33.1
Taq I	6.5, 6.2, 5.8, 4.7, 3.9, 3.1, 2.6, 1.8, 1.2, 1.0	36.8
Rsa I	2.5, 2.1, 2.0, 1.8, 1.5	9.9
Bgl II	10.5[b], 9.0[b], 7.9, 4.9, 4.1, 3.7, 2.3, 2.1[b], 1.9, 1.6, 1.4	38.3
Pvu II	8.2, 7.1, 6.6, 6.4, 5.3, 4.9, 4.3, 3.3	46.1
Pst I	13.0, 7.9, 3.0, 2.6, 2.5, 2.2, 1.9, 1.6, 1.4	36.1
Hind III	8.0, 5.9, 5.5, 4.8, 4.0, 3.8, 3.6, 3.1, 2.1, 1.1[b], 0.9[b], 0.6	42.5

[a] determined by screening 10 to 12 random genomic specimens with each restriction enzyme.
[b] variable alleles, RFLP.

Table 8 (continued): Restriction Map of Annexin III Gene

Enzyme	Bands Observed (kb)	Total kb[a]
Xba I	16.0, 9.0, 7.4, 4.9, 2.0, 1.9, 1.5	42.7
Dde I	1.0, 0.9, 0.8, 0.7, 0.5	3.9
Nco I	12.0, 10.5, 6.3, 4.6, 3.8, 2.9	40.1
Sac I	16.0, 10.5, 4.9, 4.3, 3.1, 1.4	40.2
Stu I	18, 11.0, 8.4, 4.7	42.1

[a] determined by screening one genomic specimen with each restriction enzyme.

Table 9: Restriction Map of Annexin IV Gene

Enzyme	Bands Observed (kb)	Total kb[a]
Bam HI	12.5, 11.0, 8.2, 4.5, 4.2	40.4
Eco RI	23.0, 13.0, 9.0, 7.2, 3.6	55.8
Msp I	4.4, 3.5, 2.7, 1.8, 1.6, 1.1, 1.0, 0.6, 0.5	17.2
Taq I	6.5, 4.7, 3.6, 1.5, 1.3, 1.1, 1.0, 0.7, 0.5	20.9
Rsa I	4.5, 3.0, 2.0	9.5
Bgl II	12.0, 9.0, 6.9, 3.9, 2.3, 1.6, 1.5	37.2
Pvu II	6.4, 4.4, 3.0, 2.6, 2.3, 1.7	20.4
Pst I	4.4, 3.7, 3.3, 2.5, 2.3, 2.0, 1.1	19.3
Hind III	9.4, 4.4, 2.8, 2.5, 2.1, 1.9, 1.6, 0.6, <0.6	25.9

[a] determined by screening 10 to 12 random genomic specimens with each restriction enzyme.

Table 9 (continued): Restriction Map of Annexin IV Gene

Enzyme	Bands Observed (kb)	Total kb[a]
Xba I	5.8, 5.2, 3.4, 2.5, 1.3, 1.0	19.2
Dde I	1.4, 0.5	1.9
Kpn I	20.0, 11.5	31.5
Nco I	12.5, 5.8	18.3
Sac I	23.0, 8.0	31.0
Stu I	7.9, 6.1, 5.0	19.0

[a] determined by screening one genomic specimen with each restriction enzyme.

Table 10: Restriction Map of Annexin V Gene

Enzyme	Bands Observed (kb)	Total kb[a]
Bam HI	13.0, 11.0	24.0
Eco RI	16.0, 11.0, 6.9	33.9
Msp I	14.0, 10.0, 8.0, 4.4, 2.3	38.7
Taq I	8.7[b], 7.6[b], 7.2, 6.6, 5.6[b], 2.7, 2.0, 1.6, 1.4	43.4
Rsa I	3.2, 2.2, 2.0	7.4
Bgl II	5.1, 4.6, 3.6, 2.8, 2.6, 1.8	20.5
Pvu II	10.1[b], 6.6, 6.0[b], 4.7[b], 3.7, 1.5	21.9
Pst I	7.0, 6.8, 6.1, 5.3, 4.8, 2.7, 2.5, 2.1, 1.6, 1.4, 1.3	42.7
Hind III	>23.0, 3.9, 3.3, 3.0, 1.1, 0.9, 0.7	35.9

[a] determined by screeening 10 to 12 random genomic specimens with each restriction enzyme.
[b] variable alleles, RFLP.

Table 10 (continued): Restriction Map of Annexin V Gene

Enzyme	Bands Observed (kb)	Total kb[a]
Xba I	10.0, 6.6, 3.7, 2.4, 1.3	24.0
Dde I	1.2, 0.8, 0.7, 0.5	3.2
Kpn I	16.0, 11.4	27.4
Nco I	8.8, 8.2, 5.3, 4.8	27.1
Sac I	>23.0, 2.4, 1.3	26.7
Stu I	13.0, 8.0, 5.4, 2.8	29.2

[a] determined by screening one genomic specimen with each restriction enzyme.

Table 11: RFLP Screen Conditions for Annexins III, IV, V

Enzyme	Agarose Gel	Smallest Observable Band[a]
Bam HI	0.9%	1.4 kb
Eco RI	0.7%	0.6 kb
Msp I	0.9, 1.2%	0.3 kb
Taq I	0.9, 1.4%	0.3 kb
Rsa I	0.9, 1.2%	1.4 kb
Bgl II	0.9%	1.4 kb
Pvu II	0.9%	1.4 kb
Pst I	0.7, 0.9%	0.9 kb
Hind III	0.9%	0.6 kb
Xba I	0.6, 0.9%	0.3 kb
Dde I	0.6, 0.9%	0.3 kb
Kpn I	0.6, 0.9%	0.3 kb
Nco I	0.6, 0.9%	0.3 kb
Sac I	0.6, 0.9%	0.3 kb
Stu I	0.6, 0.9%	0.3 kb

[a] detectable regions determined by the percent agarose, voltage, and time of electrophoresis.

Table 12: Annexin V RFLPs

Enzyme	Allele	Size	Frequency	Genotype	Frequency
Pvu II[a]	1	10.1[b] kb	57%	1, 1	33%
	2	6.0, 4.7 kb	43%	1, 2	47%
				2, 2	20%
Taq I[c]	1	8.7 kb	4.0%	1, 1	0.0%
	2	7.6 kb	73%	1, 2	6.0%
	3	5.6 kb	23%	1, 3	2.0%
				2, 2	57%
				2, 3	27%
				3, 3	8.0%

a based on 36 random human specimens, 72 alleles.
b 10.7 kb calculated, 10.1 kb measured.
c based on 49 random human specimens, 98 alleles.

Table 13: Annexin III RFLPs

Enzyme	Allele	Size	Frequency	Genotype	Frequency
Bgl II[a]	1	10.5[b] kb	62%	1, 1	36%
	2	9.0, 2.0 kb	38%	1, 2	51%
				2, 2	13%
Hind III[c]	1	1.1 kb	92%	1, 1	83%
	2	0.9 kb	8.0%	1, 2	17%

[a] based on 39 random human specimens, 78 alleles.
[b] 11.0 kb calculated, 10.5 kb measured.
[c] based on 24 random human specimens, 48 alleles.

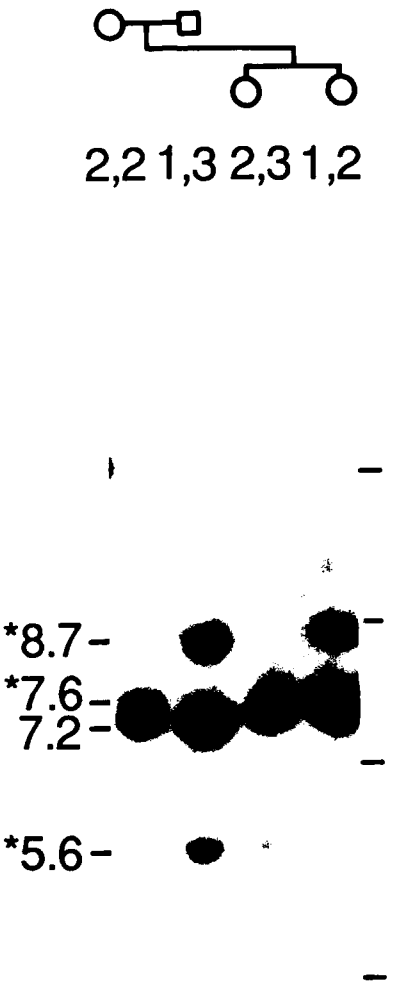

Figure 9. Taq I RFLP at the Annexin V Locus. DNA (5 ug) was digested with 4 U/ug of Taq I at 65^{o}C for 2 h and electrophoresed (40 V x 20 h) in a 0.7% agarose gel. Molecular weights of the observed bands are indicated at the left of the figure and positions of the molecular standards to the right. Polymorphic bands are indicated with **asterisks.** Assigned genotypes and family relationships are indicated at the top of the figure.

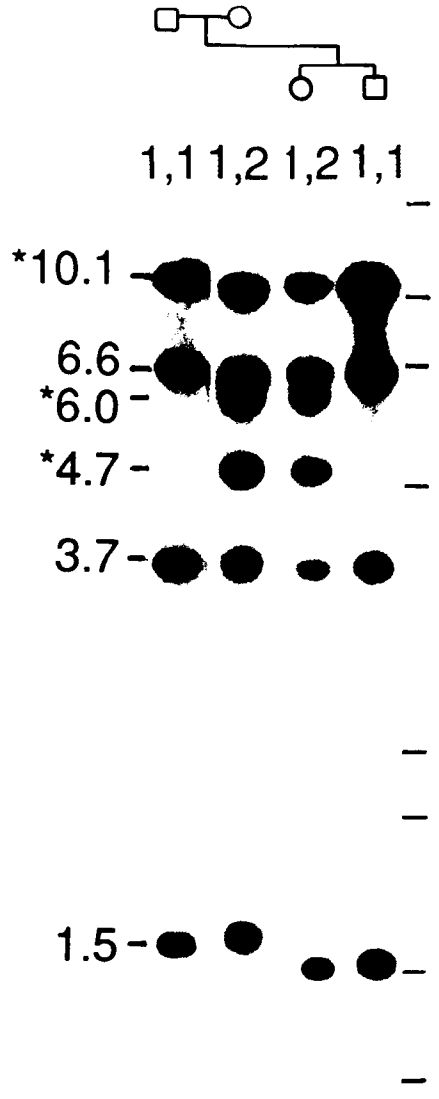

Figure 10. PVU II RFLP at the Annexin V Locus. DNA (5 ug) was digested with 6 U/ug of Pvu II at 37°C for 2 h and electrophoresed (25 V x 24 h) in a 0.9% agarose gel. Molecular weights of the observed bands are indicated at the left of the figure and positions of the molecular standards to the right. Polymorphic bands are indicated with **asterisks.** Assigned genotypes and family relationships are indicated at the top of the figure. Slight migration variation of 1.5 kb band due to composite figure of multiple gels.

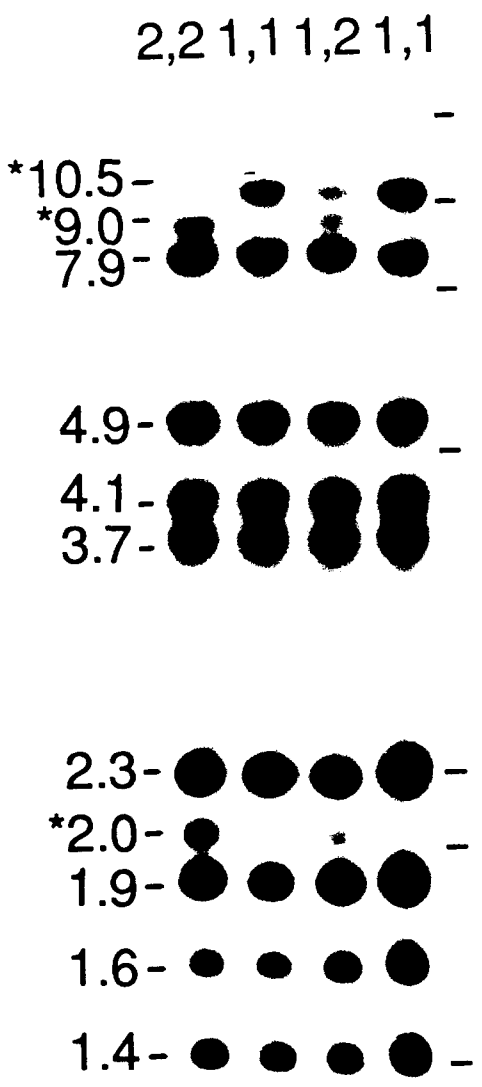

Figure 11. Bgl II RFLP at the Annexin III Locus. DNA (5 ug) was digested with 6 U/ug of Bgl II at 37°C for 2 h and electrophoresed (25 V x 25 h) in a 0.9% agarose gel. Molecular weights of the observed bands are indicated at the left of the figure and positions of the molecular standards to the right. Polymorphic bands are indicated with **asterisks.** Assigned genotypes are indicated at the top of the figure.

CHAPTER IV

Discussion and Conclusions

Chromosomal Localization

The gene for annexin III is located on chromosome 4. The localization was identified by three independent techniques: PCR of intron sequence using somatic cell hybrids (Figure 5), Southern blot of DNA restriction enzyme digests (Figure 7), and in-situ hybridization (performed in collaboration with Drs. David Adler and Christine Disteche) to chromosome 4q21.

The gene for Annexin V is located on chromosome 4. This chromosome was identified by two independent techniques: PCR of intron sequence using somatic cell hybrids (Figure 2), and in-situ hybridization (performed in collaboration with Drs. David Adler and Christine Disteche) to 4q27. The localization of annexin V to chromosome 4 confirms previous localization of annexin V to 4q28-32 (Modi et al., 1989). Their localization was performed using Southern blot analysis of somatic cell hybrids and in-situ hybridization.

The chromosomal localization results for annexin IV using PCR and Southern blot analysis were inconclusive. However, in-situ hybridization (performed in collaboration with Drs. David Adler and Christine Disteche) demonstrated a unique locus at 2p13.

Failure to localize annexin IV using Southern blot evaluation of the Hind III digests of human genomic DNA is interesting since Hind III was selected as the enzyme of choice for Southern blot analysis of annexin IV. Possible reasons for the inability to identify the characteristic human restriction pattern might be due to the relatively small amount of DNA used or to a variable percentage of the gene of interest present in each cell line. Hybrid cell lines containing a low number of copies of the annexin IV gene may have escaped detection with hybridization of the cDNA probe.

The percent discordancy obtained with PCR analysis for the annexin IV gene in the hybrid cell lines ranged from 8% to 76%, with chromosome 2 showing the least discordance. Repeat performance of the PCR reactions with the cell lines produced inconsistent results. These sporadic results may be due to the large size (3.5 kb) of the PCR product amplified. Large PCR products (greater than 1.5 kb) are less consistently amplified in the PCR reaction.

The PCR parameters used for localization of annexin IV were similar to those used for annexin III and V with two exceptions: 40 cycles for amplification of products instead

of 30, and 2.0 mM Mg^{2+} in place of 1.5 mM Mg^{2+}. The amplified products seen in cell lines lacking chromosome 2 (983 and 904) could have been produced from genes closely related to annexin IV. The additional 10 cycles of amplification may have produced enough product, although less product than the cell line containing chromosome 2 (854), to show weak hybridization on the Southern blot.

As mentioned in Chapter III, the increased $MgCl_2$ concentration was used to increase synthesis of the desired product. However, increasing the Mg^{2+} concentration can also decrease specificity of amplification (Ehrlich, 1989). Quite possibly the optimal Mg^{2+} concentration was reached and slightly exceeded, thereby allowing amplification of other products from a related gene or an unrelated gene oriented perfectly in cell lines 983 and 904 to allow amplification.

Chromosome 2 may be present in low concentration in cell lines 983 and 904. This would be similar to the presence of chromosome 4 in cell line 867 discovered during chromosomal localization of annexin V.

Failure to localize annexin V using Southern blot evaluation of the Hind III digests of human genomic DNA is interesting since annexin III localization results correlated perfectly with the PCR results. Possible explanations for this localization failure include the two reasons stated for annexin IV, the fact that Hind III is not the optimal enzyme for producing different sized

inter-species restriction fragments with annexin V, and the fact that the annexin III and IV probes were stripped from the membranes prior to the hybridization with annexin V cDNA probe. Stripping of the membrane may have removed just enough DNA to prevent localization with hybridization of the cDNA probe. However, the more sensitive technique of chromosomal localization by PCR proved successful.

Restriction Fragment Length Polymorphism (RFLP) Study

As outlined in Chapter I, RFLPs serve a very important function in the mapping of the human genome and diagnosis of genetic disease. At the present time the RFLPs associated with the annexin family do not have any direct clinical application. However, as more is discovered about the in-vivo role of the annexins, the chromosomal location of all members, and the identification of genes in close proximity to the annexins, the value of existing RFLPs will increase. Nonetheless, the RFLPs identified in this research project are by themselves very interesting (Table 12 & Table 13).

Chromosomal Localization Techniques

Chromosomal localization based on PCR amplification of somatic cell hybrids is a very new technique, with only a handful of published studies so far (Iggo et al., 1989; Dionne et al., 1990). Application in this project proved very successful. This technique can be performed without knowledge of specific gene structure (Iggo et al., 1989).

Two pieces of information were used to make educated guesses about intron locations in the annexin genes: the location of the repeating units in the proteins and the structure (including intron-exon boundaries) of the murine annexin II gene (Amiguet et al., 1990). Primers were designed with certain specifics in mind: primer pairs should be of similar length, primer pairs should have approximately the same GC content, and primers should not have complementary sequences at the 3' end. Once primer pairs successfully amplified product larger than the product from the cDNA, PCR conditions were optimized for each primer combination. Multiple thermal profiles were tested with each primer pair to ensure that the best intensity and specificity of amplified products was obtained. The PCR parameters adjusted in this study included amount of DNA template, denaturation time and temperature, annealing time and temperature, extension time and temperature, the concentration of $MgCl_2$ in the PCR buffer, and number of amplification cycles.

The technique of chromosomal localization using hybrid cell lines based on PCR amplification of intron sequence holds some advantages over chromosomal localization via amplification of exon sequence. The absence of the human-specific PCR product using amplification of exon sequence could be due to the absence of the gene(s) containing the complementary sequence or due to failure of the PCR reaction. Failure of the PCR reaction may be due to a

myriad of problems unrelated to the presence of the genetic region of interest, thus producing false negative results.

Chromosomal localization utilizing intron amplification relies on the presence of a human PCR product(s) of a specific size, along with product(s) of different sizes amplified from similar or homologous sequence in the hybrid DNA, or no amplificiation of hybrid PCR products. Hybrid cell lines lacking the human specific PCR products but producing the hamster PCR products, almost certainly do not contain the human gene of interest. Thus, false negative results due to the absolute failure of the PCR reaction are ruled out. Chromosomal localization based on PCR amplification of intron sequence includes another level of quality control. Since intron sequence is not highly conserved from species to species, PCR products of identical size are usually not amplified from two different species. Therefore, production of human-sized product in hybrid cell line suggests human origin.

As the PCR results with annexin V showed, primers will sometimes amplify sequences of a different size in dissimilar species (Figure 2). These amplified sequences may be the hamster annexin V gene, other known annexin genes, other unknown annexin genes, or totally unrelated genes. By contrast, annexin III PCR reactions did not amplify discernable PCR product with hamster genomic DNA (Figure 5). This difference is most likely due to a

decreased degree of complementarity between the human derived primers and hamster exon sequence.

Three virtues of chromosomal localization via PCR amplification of intron sequence are: it is more rapid than the Southern blot technique, the procedure is non-radioactive provided the product is visible on the ethidium bromide stained gel, and it can be performed with very small quantities of relatively impure template DNA (Iggo et al., 1989).

Preparation time for the more conventional chromosomal localization technique, Southern blots of restriction digests of genomic DNA, is often as long as 3 to 5 days. Chromosomal localization via PCR amplification of intron sequence, at least the initial detection on ethidium bromide stained gels, can be completed in one day.

PCR products indicating initial chromosomal localization of annexin III and V were visible on the ethidium bromide stained gels. Southern blotting was utilized for confirmation of these PCR products for annexins III and V. In comparison, Southern blots of PCR products from all 25 hybrid cell lines were used for initial detection of gene origin for annexin IV. These Southern blots were performed for all cell lines since PCR products were not visible on ethidium bromide stained agarose gels.

The use of PCR for chromosomal localization brings an increased degree of sensitivity when compared to the more traditional method of Southern blot evaluation of genomic

DNA digested with restriction enzymes. This increased sensitivity, due to the exponential production of the specific template sequence, affords the luxury of using significantly less DNA (Saiki et al., 1988). 4 ug to 9 ug of genomic DNA in preparing the Hind III Southern blots used for localization of the three annexin proteins. In contrast, the amount of template DNA used in the PCR localization assays varied from 100 ng to 500 ng. The amplified PCR product is largely target sequence including any intron sequence located between the primer sites. The genomic specimens cleaved with restriction enzyme contain an infinitesimally (close to one in a million based on cDNA size) small percentage of target sequence compared to total DNA.

Future Work

Where does the project go from here? Future work on this project lies in a few basic areas. In order to verify that these proteins were localized to their chromosome of origin, the exact base sequence of the PCR products could be obtained. If correct, sequenced amplified products would contain regions identical to the cDNA sequences separated by intervening sequences, introns.

Verification of chromosome 2 as the origin of annexin IV should be performed with an alternate hybrid cell line containing chromosome 2 as its only human chromosome. This chromosomal localization, using PCR amplification of intron

sequence, should clarify the discordant results seen with the human-hamster somatic cell hybrids. An alternative plan would be to design new PCR primers that would amplify a product smaller than 3.5 kb. This smaller product should be amplified more consistently.

Characterization of the entire genes coding for these three annexins would be another worthwhile project. Information about annexin function and regulation of gene expression would undoubtedly be obtained.

The RFLP study could be expanded by the digestion of human genomic DNA with additional enzymes. These additional restriction patterns and possible RFLPs would provide further insight into annexin gene structure and disease linkage.

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